JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1994, p. 2528–2532
0095-1137/94/$04.00+0

Vol. 32, No. 10

# Monitoring Spread of *Malassezia* Infections in a Neonatal Intensive Care Unit by PCR-Mediated Genetic Typing

ALEX VAN BELKUM,[1]* TEUN BOEKHOUT,[2] AND RON BOSBOOM[3]

*Department of Molecular Biology, Diagnostic Center SSDZ, 2600 GA Delft,[1] Yeast Division, Centraalbureau voor Schimmelcultures, 2628 BC Delft,[2] and Department of Medical Microbiology, Academic Hospital Leiden, 2300 RC Leiden,[3] The Netherlands*

Received 4 March 1994/Returned for modification 20 June 1994/Accepted 14 July 1994

***Malassezia furfur* and *Malassezia pachydermatis* were isolated from newborn children and incubators in a neonatal intensive care unit. To assess whether persistence or frequent import of the organisms was the cause of the elevated incidence, genetic typing of the strains was performed by PCR-mediated DNA fingerprinting. By using PCR primers aimed at repeat consensus motifs, six different genotypes could be detected in a collection of six *M. furfur* reference strains. In the case of 10 *M. pachydermatis* reference strains, nine different genotypes were detected by three different PCR assays. None of these assays could document genetic differences among the clinical isolates of either *M. furfur* or *M. pachydermatis*. On the basis of these results it is concluded that within the neonatal intensive care unit the longitudinal persistence of both an *M. furfur* and an *M. pachydermatis* strain has occurred and that *Malassezia* species can persist on incubator surfaces for prolonged periods of time. It can be concluded that PCR fingerprinting is a *Malassezia* typing procedure that is to be preferred over the analysis of chromosomal polymorphisms by pulsed-field gel electrophoresis in this genus.**

The basidiomycetous yeast genus *Malassezia* presently consists of three species (19). *Malassezia furfur* and *Malassezia sympodialis* are obligatory lipophilic skin flora yeasts of humans, whereas *Malassezia pachydermatis* is a nonobligatory skin flora yeast also encountered in several other mammals (12). *M. furfur* can cause a relatively broad spectrum of clinical phenomena, varying from pityriasis versicolor in adults (1) to life-threatening invasive disease in neonates (7, 11, 13, 16). There is also an increasing number of reports discussing the relevance and potential clinical problems of *Malassezia* infections in cancer (8, 18) or AIDS patients (19). Since *Malassezia* species are being encountered in clinical diagnostics more frequently, the need for identification and typing assays will increase in the coming years.

For *M. furfur*, an immunological assay discriminating three serovars has been described (6). It is clear that this degree of resolution is insufficient for detailed epidemiological comparisons among clinical and environmental isolates. For epidemiological typing of yeasts in general various molecular procedures have been developed (15), but with respect to *Malassezia* typing only a small number of reports have appeared in the literature. These mainly elaborate on karyotyping by pulsed-field gel electrophoresis (3, 10), which again does not allow for the highly efficient discrimination of *Malassezia* strains. In order to discriminate the persistence of *Malassezia* strains from increased importation, novel procedures must be implemented. In this respect PCR may prove to be valuable (14, 20). PCR can be used for the detection of subtle DNA polymorphisms by application of primers that aim at hypervariable DNA loci (23, 24). This laboratory technique has already been used frequently in order to determine interisolate relationships among fungal and protozoal species (9, 20–22). The present report describes the application of PCR fingerprinting for the resolution of *Malassezia* epidemics in a neonatal intensive care unit. Recently, within a neonatal intensive care unit at the Academic Hospital Leiden (Leiden, The Netherlands), an increase in the number of *M. furfur* and *M. pachydermatis* infections was encountered (5). Several of the environmental and patient isolates were typed by PCR, and the epidemiological implications will be discussed. Since some of the strains have also been karyotyped by pulsed-field electrophoretic separation of chromosomes (3), a comparison between karyotyping and PCR genotyping could be performed.

## MATERIALS AND METHODS

**Yeast strains and pediatric patients.** In the present study *M. furfur*, *M. pachydermatis*, and *M. sympodialis* strains were analyzed. *M. furfur* and *M. pachydermatis* were grown on Leeming and Notman agar (1% peptone, 0.5% glucose, 0.1% yeast extract, 0.4% desiccated ox bile, 0.1% glycerol, 0.05% glycerol monostearate, 0.05% Tween 60, 1% whole-fat cow's milk, 1.5% agar). In the case of *M. furfur* cultivation, this medium was supplemented with a small amount of olive oil. *M. sympodialis* was grown on 1% yeast extract, 0.5% peptone, 4% glucose agar (YPGA medium). All strains studied were cultivated at 35°C (Table 1). Table 1 lists the reference isolates from the Centraalbureau voor Schimmelcultures (CBS; Yeast Division, Delft, The Netherlands) and the clinical isolates collected in the departments of neonatology or dermatology at the Academic Hospital Leiden. Relevant patient data are included in Table 1.

**DNA isolation and PCR.** To prevent contamination in the PCR assays, DNA isolation and DNA amplification were performed in separate laboratory rooms. Transport of laboratory equipment between these rooms was prohibited. Also, the PCR ingredients were stored in a separate room. In order to isolate DNA, cells were scraped from the culture media and were collected in an appropriate volume of phosphate-buffered saline (PBS; pH 7.0). Cells were pelleted by centrifugation, washed with another volume of PBS, and centrifuged again. For the *M. pachydermatis* and *M. sympodialis* strains, effective lysis could be achieved by treating cells with Novozym (Novo

* Corresponding author. Mailing address: Department of Clinical Microbiology, Academic Hospital Dijkzigt, Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands.

TABLE 1. Overview of *Malassezia* strains examined in the study

| Organism | Strain[a] | Source |
|---|---|---|
| *M. furfur* | 1. CBS 1878 | R. W. Benham, type of *Pityrosporum ovale* |
| | 2. CBS 7019 | V. K. Hopsu, neotype, pityriasis versicolor, Finland |
| | 3. CBS 6094 | F. Keddie, normal skin, United States |
| | 4. CBS 4172 | B. A. Custafson, skin of eland |
| | 5. CBS 5333 | F. Flank, lesion of skin, Canada |
| | 6. CBS 6000 | I. G. Murray, ex dandruff, India |
| | 7. AZL 113598 | R. Bosboom, urine of neonate B, 11-5-90[b] |
| | 8. AZL 112926 | R. Bosboom, urine of neonate A, 6-5-90 |
| | 9. AZL 115292 | R. Bosboom, urine of neonate B, 28-5-90 |
| | 10. AZL 132977 | R. Bosboom, urine of neonate D, 26-11-90 |
| | 11. AZL 133102 | R. Bosboom, urine of neonate E, 27-11-90 |
| | 12. AZL 112426 | R. Bosboom, skin of neonate A, 30-4-90 |
| | 13. AZL 113850 | R. Bosboom, urine of neonate B, 13-6-90 |
| *M. sympodialis* | 14. CBS 7222 | R. B. Simons, auditory tract, United States |
| | 15. CBS 7709 | E. Guley, skin with seborrheic eczema, The Netherlands |
| | 16. CBS 7705 | E. Guley, pityriasis versicolor, The Netherlands |
| | 17. CBS 7707 | E. Guley, pityriasis versicolor, The Netherlands |
| *M. pachydermatis* | 18. CBS 7044 | R. van Breuseghem |
| | 19. CBS 4165 | |
| | 20. CBS 1891 | J. Lodder, ear of dog, The Netherlands |
| | 21. CBS 1884 | B. A. Gustafson, ear of dog, Sweden |
| | 22. CBS 6534 | H. E. Rhoades, ear of dog, United States |
| | 23. CBS 1919 | P. W. C. Austwick, ulcerated ear of dog, United Kingdom |
| | 24. CBS 6541 | H. E. Rhoades, ear of dog |
| | 25. CBS 6537 | H. E. Rhoades, ear of dog, United States |
| | 26. CBS 1885 | J. Lodder, ear of dog, The Netherlands |
| | 27. CBS 6542 | H. E. Rhoades, ear of dog |
| | 28. CBS 6535 | H. E. Rhoades, ear of dog, United States |
| | 29. AZL J | 19-3-91 |
| | 30. AZL A4 | Incubator, 22-1-91 |
| | 31. AZL W | 15-1-91 |
| | 32. AZL D5 | Incubator, 27-3-91 |
| | 33. AZL KV | 17-4-91 |
| | 34. AZL 107498 | NK[c] |
| | 35. AZL RV | 26-2-91 |
| | 36. AZL SH | 19-3-91 |
| | 37. AZL 101992 | NK |
| | 38. AZL ST | 22-1-94 |
| | 39. AZL D3 | Incubator, 6-3-91 |
| | 40. AZL CM | 17-4-91 |
| | 41. AZL WS | 5-2-91 |
| | 42. AZL JV | 17-1-91 |

[a] CBS, Yeast Division, Centraalbureau voor Schimmelcultures, Delft, The Netherlands; AZL, Academic Hospital Leiden, Leiden, The Netherlands. The numbers 1 through 42 correspond to the numbering used in Fig. 1 and 2.
[b] Isolation dates are given in day-month-year.
[c] NK, date of isolation not known.

Industries, A/S), subsequent spheroplast lysis, and DNA purification by a method with guanidinium isothiocyanate (4). Since the clinical *M. furfur* isolates seemed to resist this procedure, an alternative and more generally applicable procedure for the isolation of DNA from *Malassezia* species was developed. Cell pellets were washed once more in distilled water and lyophilized for at least 60 h. The resulting powder was resuspended in a buffer containing 100 mM Tris-HCl (pH 6.4), 4 M guanidine isothiocyanate, 10 mM EDTA, and 2% (vol/vol) Triton X-100, and the mixture was incubated at 37°C for at least 2 h. Thereafter DNA was purified by affinity chromatography (22). The amounts of isolated DNA were estimated by electrophoresis in 1% agarose gels in 40 mM Tris-borate (pH 7)–1 mM EDTA (0.5× TBE) and comparison with ethidium bromide-stained amounts of bacteriophage lambda DNA (Promega) in a parallel run.

For PCR, approximately 50 to 100 ng of *Malassezia* DNA was dissolved in a 100-μl volume containing 10 mM Tris-HCl (pH 9), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 0.2 mM (each) deoxyribonucleotide triphosphate, and 0.5 U of *Taq* DNA polymerase (Sphaero Q, Leiden, The Netherlands). The following primers (sequences) were used for PCR fingerprinting of *Malassezia* strains: ERIC IR (5′-ATGTAAGCTCCTGGGGATTCAC-3′), ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′), REP1R-I (5′-IIIICGICGICATCTGGG-3′), REP2-I (5′-ICGICTTATCIGGCCTAC-3′), and BG-2 (5′-TACATTCGAGGACCCCTAAGTG-3′). (I stands for the base derivative inosine). All primers have been described previously (22, 23). After the addition of 50 pmol of one or more of the PCR primers, the aqueous phase was overlaid with mineral oil and thermocycling was performed in a Biomed type 60 PCR machine. The PCR program consisted of a 4-min predenaturation step at 94°C; 40 cycles of 1 min at 94°C, 1 min at 25°C, and 2 min at 74°C; and a final

extension step of 4 min at 74°C. Amplimers were analyzed by electrophoresis through 1 to 3% agarose gels in 0.5× TBE. Ethidium bromide-stained gels were photographed by using Polaroid Polapan T52 films.

PCR results were validated only when the negative control samples (in which no extraneous DNA was included) were demonstrated not to contain DNA after PCR.

## RESULTS

All strains listed in Table 1 were subjected to various PCR assays. Initial experiments with the arbitrary primer BG2 (22) appeared to be unsuccessful, although this primer has been very useful in elucidating the interrelationships among isolates of other fungal species. The gels showed monomorphic DNA banding patterns of low complexity because only a few DNA fragments were synthesized (data not shown). Pilot experiments involving a small number of *Malassezia* strains revealed that the application of single REP or ERIC primers (23) was not highly discriminatory. However, the combination of primers ERIC IR and ERIC2 in a single reaction revealed a sufficiently high degree of DNA diversity among the various strains. As can be seen in Fig. 1, all six CBS strains of *M. furfur* could be unequivocally identified. In the group of 10 CBS strains of *M. pachydermatis*, nine different types were detected. In Table 2, the *M. pachydermatis* genocodes constructed by identifying given DNA fingerprints with a letter or a number show that the combined results of two PCR assays allowed 9 of 10 strains to be discriminated. Only CBS 6541 and CBS 6535, both U.S. isolates originating from the dog auditory tract, could be discriminated. In order to gain insight into the genetic diversity that can be highlighted by PCR fingerprinting of this *Malassezia* species, more isolates, preferably from multiple geographic origins, must be analyzed.

All *M. sympodialis* reference strains could be distinguished on the basis of their DNA fingerprints. It was shown that the banding pattern of CBS 7222, the type strain of *M. sympodialis*, was grossly different from those of the other strains. All clinical *M. pachydermatis* strains appeared to be identical (Fig. 1). Interestingly, all incubator isolates (AZL A4, AZL D5, and AZL D3) were genetically comparable. This implies that despite regular cleaning of the incubators, *M. pachydermatis* persists on the glass surfaces. Since the isolation dates were over 2 months apart, this persistence may be clinically relevant. Apparently, the clinical *M. furfur* strains are clonally related, since strains AZL 115292 and AZL 133102 displayed minor differences with regard to the other hospital isolates when the combination of the ERIC primers was used. These slightly abberant strains were derived from two different patients. The differences among the clinical isolates of *M. furfur* are much smaller than those among the CBS reference strains. The combination of REP1R-I and REP2-I primers generated useful results, especially for the *M. pachydermatis* strains. However, PCR fingerprints of *M. furfur* and *M. sympodialis* were relatively vague, but the results described above were supported. PCR with REP1-REP2 did not discriminate among the clinical *M. furfur* strains. Figure 2 shows the results for *M. pachydermatis* type strains and clinical isolates.

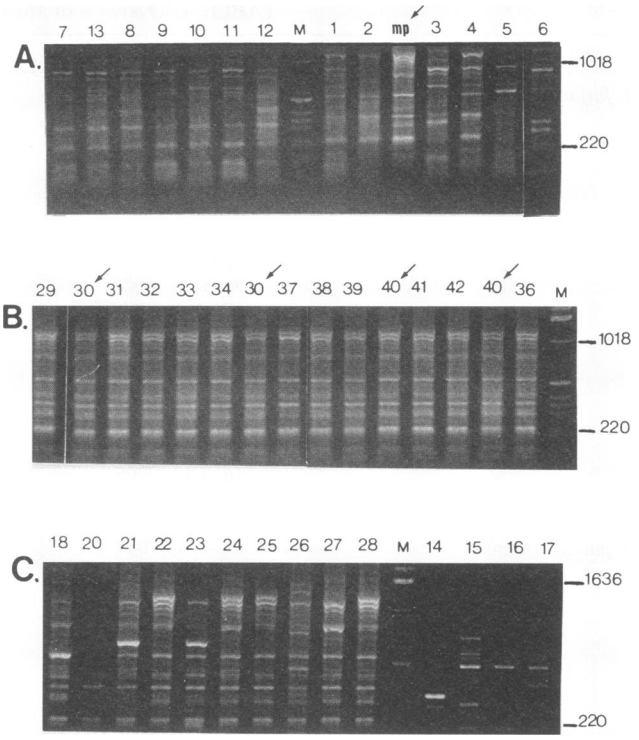


FIG. 1. Typing of *Malassezia* strains by PCR fingerprinting with primers ERIC IR and ERIC2. (A) Comparison of clinical isolates (strains 7 to 13) and reference strains (strains 1 to 6) of *M. furfur*. The banding patterns indicate homogeneity among the clinical isolates versus genetic heterogeneity among the reference strains. Note that clinical isolates 9 and 11 displayed minor differences when they were compared with the other patient strains (see also Results). The isolate indicated mp (arrow) represents *M. pachydermatis* (strain 28). (B) PCR analysis of clinical *M. pachydermatis* strains. Strains 30 and 40 were run in duplicate (arrows). All isolates display identical DNA banding patterns. (C) PCR analysis of *M. pachydermatis* type strains (strains 18 and 20 to 28). DNA isolated from strain 20 appeared to be of poor quality. Strains 14 to 17 were *M. sympodialis*; strain 14 gave a clearly abberant DNA banding pattern. Molecular mass markers (M) are indicated on the right (in base pairs). Numbering of strains is as in Table 1.

## DISCUSSION

Whereas several genomic typing procedures for *Malassezia* yeasts appeared to be insufficiently discriminatory, PCR fingerprinting can be successfully used for the adequate typing of *Malassezia* strains. Although the isolation of DNA from some strains may be troublesome and not all PCR primers generate sufficiently variable DNA banding patterns, several epidemiologically useful assays can be developed by including multiple, prokaryotic repeat consensus primers like the ERIC or REP motifs. DNA can be isolated successfully by combining lyophilization with the lytic activity of the chaotropic salt guanidine isothiocyanate. Sufficiently discriminative assays can be designed.

As demonstrated in this report, on the basis of the homogeneity of the fingerprints of all clinical *Malassezia* isolates, as opposed to the variation observed in groups of reference strains, it must be concluded that in the neonatology ward of the Academic Hospital Leiden genuine nosocomial epidemics of both *M. furfur* and *M. pachydermatis* have occurred. This is corroborated by the fact that the *M. pachydermatis* isolates found to colonize the incubators were genetically indistinguishable over time. Also, *M. furfur* could be found on incubator surfaces. For this species it has been demonstrated that genotypically homologous strains could be isolated from the same surfaces 3 months after the original culture was obtained (data not shown). This phenomenon indicates that regular

TABLE 2. PCR fingerprinting genocodes for CBS strains of *M. pachydermatis*

| CBS strain | Strain no. in Table 1 and Fig. 1 and 2 | Type by PCR assay with[a]: | | Overall type[b] |
|---|---|---|---|---|
| | | ERIC IR-ERIC2 | REP1R-I REP2-I | |
| CBS 7044 | 18 | A | A | I |
| CBS 1891 | 20 | B | B | II |
| CBS 1884 | 21 | C | C | III |
| CBS 6534 | 22 | D | D | IV |
| CBS 1919 | 23 | E | E | V |
| CBS 6541 | 24 | F | F | VI |
| CBS 6537 | 25 | D | F | VII |
| CBS 1885 | 26 | G | G | VIII |
| CBS 6542 | 27 | H | H | IX |
| CBS 6535 | 28 | F | F | VI |

[a] Both assays identified eight types within the group of 10 strains. Combination of the results left only strains CBS 6541 and CBS 6535 undiscriminated.
[b] Nine types were identified overall.

hygienic measures do not adequately remove or kill *Malassezia* yeasts.

Several of the strains described in this report were karyotyped by pulsed-field gel electrophoresis (3). From the results of these studies it appears that the species *M. pachydermatis*, *M. furfur*, and *M. sympodialis* could be identified on the basis of their characteristic karyotypes, also showing that all species harbor a relatively small genome when compared with the genome sizes of other yeast species (25). Neither of the clinical *M. furfur* isolates could be discriminated on the basis of chromosomal length polymorphism, whereas the different CBS type strains of *M. furfur* could be divided into four distinguishable karyotypes. This type of DNA heterogeneity is in full agreement with the PCR results. The results obtained for *M. pachydermatis* seem to be contradictory. On the basis of the length polymorphisms found in the smallest chromosomes, it has been suggested that the clinical isolates are diverse and that reinfection rather than an epidemic has taken place in the neonatology ward (3). The PCR fingerprinting results, however, seem to indicate a genuine epidemic. In order to explain this contradiction, it could be assumed that the chromosomal length variation observed in the clinical *M. pachydermatis* isolates is due to copy number variation in the repetitive ribosomal operons. This corroborates previously observed phenomena which reflect only a minor single-locus variability, which is generally considered a result of growth conditions.

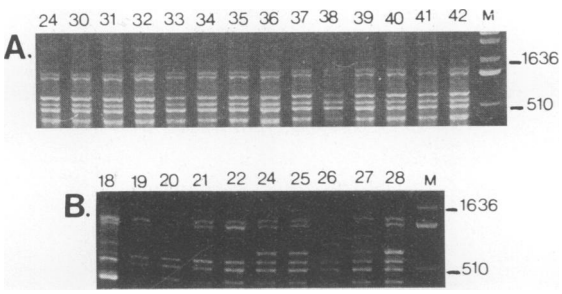


FIG. 2. Typing of *M. pachydermatis* strains by PCR fingerprinting with primers REP1R-I and REP2-I. All clinical isolates appeared to be identical (A), whereas heterogeneity was encountered among the type strains (B). Molecular mass markers are indicated on the right (in base pairs). For indexing of the banding patterns for the type strains, see Table 2.

Amplification of ribosomal operons as a consequence of environmental factors has been demonstrated before (2, 17) and cannot be used as a marker for overall genetic variability. The more dispersed type of variation (multilocus) detected by PCR may give a more global picture of genomic evolution. As a consequence the results of PCR fingerprinting of *Malassezia* DNA may have more extensive epidemiological value than the detection of chromosomal length variation by pulsed-field gel electrophoresis.

## REFERENCES

1. **Akpata, L. E., H. C. Gugnoin, R. Srivastava, and S. J. Utsalo.** 1992. Dermatomycoses among industrial workers in Cross River State, Nigeria. Mycoses **35**:371–374.
2. **Asakura, K., S. Iwaguchi, M. Homma, T. Sukal, K. Higashide, and K. Tanaka.** 1991. Electrophoretic karyotypes of clinically isolated yeasts of *Candida albicans* and *C. glabrata*. J. Gen. Microbiol. **137**:2531–2538.
3. **Boekhout, T., and R. W. Bosboom.** 1994. Karyotyping of *Malassezia* yeasts: taxonomic and epidemiological implications. Syst. Appl. Microbiol. **17**:146–153.
4. **Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. Wertheim-van Dillen, and J. Van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28**:495–503.
5. **Bosboom, R. W., G. W. Van der Kamp, and P. L. G. Petit.** 1993. *Malassezia* spp. epidemic in a neonatal intensive care unit, abstr. 509. Proc. Abstr. VI Eur. Congr. Clin. Microbiol. Infect. Dis., Seville, Spain.
6. **Cunningham, A. C., E. Ingham, and G. Gowland.** 1992. Humoral response to *Malassezia furfur* serovars A, B and C in normal individuals of various ages. Br. J. Dermatol. **127**:476–481.
7. **Dankner, W. M., S. A. Spector, J. Furer, and C. E. Davis.** 1987. *Malassezia* fungemia in neonates and adults: complication of hyperalimentation. Rev. Infect. Dis. **9**:743–753.
8. **Francis, P., and T. J. Walsh.** 1992. Approaches to management of fungal infections in cancer patients. Oncology **6**:133–148.
9. **Kersulyte, D., J. P. Woods, E. J. Keath, W. E. Goldman, and D. E. Berg.** 1992. Diversity among clinical isolates of *Histoplasma capsulatum* detected by polymerase chain reaction with arbitrary primers. J. Bacteriol. **174**:7075–7079.
10. **Kiuchi, A., S. Takaraguchi, R. Hanizawa, M. Hara, T. Ikeda, and K. Tabuci.** 1992. Chromosome sized DNA of *Malassezia pachydermatis* by pulsed field gel electrophoresis. J. Vet. Med. Sci. **54**:1219–1220.
11. **Larocco, M., A. Dorenbaum, A. Robinson, and L. K. Pickering.** 1988. Recovery of *Malassezia pachydermatis* from eight infants in a neonatal intensive care nursery: clinical and laboratory features. Pediatr. Infect. Dis. J. **7**:398–401.
12. **Marcon, M. J., and D. A. Powell.** 1992. Human infections due to *Malassezia* spp. Clin. Microbiol. Rev. **5**:101–119.
13. **Mickelsen, P. A., M. C. Viano-Paulson, D. A. Stevens, and P. S. Diaz.** 1988. Clinical and microbiological features of infection with *Malassezia pachydermatis* in high risk infants. J. Infect. Dis. **157**:1163–1168.
14. **Persing, P. H., and M. L. Landry.** 1989. In vitro amplification techniques for the detection of nucleic acids: new tools for the diagnostic laboratory. Yale J. Biol. Med. **62**:159–171.
15. **Pfaller, M. A.** 1992. The use of molecular techniques for epidemiologic typing of *Candida* species. Curr. Top. Med. Mycol. **4**:43–63.
16. **Richet, H. M., M. M. McNeil, M. C. Edwards, and W. R. Jarvis.** 1989. Cluster of *Malassezia furfur* pulmonary infections in a neonatal intensive care unit. J. Clin. Microbiol. **27**:1197–1200.
17. **Rustchenko-Bulgac, E. P.** 1991. Variations of *Candida albicans* electrophoretic karyotypes. J. Bacteriol. **173**:6586–6596.
18. **Samonis, G., and D. Baljavoukas.** 1992. Fungal infections in cancer patients: an escalating problem. In Vivo **6**:183–193.
19. **Simmons, R. B., and E. Gueho.** 1990. A new species of *Malassezia*. Mycol. Res. **94**:1146–1149.
20. **Van Belkum, A.** 1994. DNA fingerprinting of medically important microorganisms by use of PCR. Clin. Microbiol. Rev. **7**:174–184.

21. **Van Belkum, A., J. F. De Jonckheere, and W. G. V. Quint.** 1992. Genotyping of *Naegleria* spp. and *N. fowleri* isolates by interrepeat PCR. J. Clin. Microbiol. **30:**2595–2598.
22. **Van Belkum, A., W. G. V. Quint, B. De Pauw, W. Melchers, and J. F. G. M. Meis.** 1993. Typing of *Aspergillus* species and *Aspergillus fumigatus* isolates by interrepeat PCR. J. Clin. Microbiol. **31:**2502–2505.
23. **Versalovic, J., T. Koeuth, and J. R. Lupski.** 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. **19:**6823–6831.
24. **Welsh, J., and M. McClelland.** 1991. Genomic fingerprinting using arbitrarily primed PCR and a matrix of pairwise combination of primers. Nucleic Acids Res. **40:**275–279.
25. **Yoganathan, T., H. Lind, and G. A. Buck.** 1989. An electrophoretic karyotype and assignment of ribosomal genes to resolved chromosomes of *Pneumocystis carinii*. Mol. Microbiol. **3:**1473–1480.